

**10/588095****IAP5 Rec'd PCT/PTO 28 JUL 2006****【DESCRIPTION】****【Invention Title】**

**THE USAGE OF MADS-BOX GENES IN FRUIT & SEED  
DEVELOPMENT BY REGULATING ACTIVE GIBBERELLIN SYNTHESIS**

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**【Technical Field】**

The present invention relates to a novel use of a MADS-box gene. In particular, it relates to the use of a MADS-box gene selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain, for the regulation of fruit and seed development.

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**【Background Art】**

In the most of angiosperm plants, fruit provides a suitable environment for seed maturation and often a mechanism for the dispersal of mature seeds.

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The Fruit development usually starts with development and fertilization of ovary, which occurs by a variety of chemical and physiological changes (Dong Y-H et al., J. Amer. Soc. Hort. Sci., 1997, 122:752-757). After

the completion of fertilization, an egg cell develops into an embryo, polar nuclei develop into endosperm and an ovule develops into seed. An ovary develops into fruit by the differentiation and growth of a cell and transformation and accumulation of reserve substances therein. However, there are some cases where not only an ovary but also other parts of a floral organ such as receptacle are involved in the fruit development. Such fruits are called pome fruits and an apple is an example.

10 In general, the development of ovary into fruit is also accompanied by a seed formation, however, in the case of fruits without seed, seed does not form even after the fertilization and only the ovary itself develops. The forming a fruit without seed is called parthenocarpy.

15 Parthenocarpy occurs when ovary develops into a fruit in the absence of pollination (citrus, banana, pineapple, etc) or fertilization of an egg cell after pollination (some orchidaceae), or an embryo is aborted after the fertilization (grapes, peach, cherry etc).

20 MADS-box genes represents a gene family composing 30 or more amino acid sequences encoding a transcription factor containing a conserved region called MADS-domain. Many of those genes have been shown to be involved in the differentiation of a flower and other various organs in a

25 plant by regulating transcription. Specifically, MADS-box

genes are classified into group A, group B and group C, according to their respective functions (ABC model). Genes of group A take responsible for the development of sepal and petal, genes of group B for the development of petal and stamen, and genes of group C for the development of stamen and pistil. Among MADS-box genes derived from *Arabidopsis thaliana*, *APETALA1* (AP1) belongs to group A, *APETALA* (AP3) and *PISTILL* (PI) have been confirmed to belong to group B, and *AGAMOUS* (AG) is classified into group C (Gunter Theissen et al., *Plant Molecular Biology*, 2000, 42: 115-149). Further, *Floral binding protein 7* (FBP7) and *floral binding protein 11* (FBP11) which are found to be involved in the development of ovule have been classified into new group D (Gerco C. Angenent et al., *Plant Cell*, 1995, 7: 1569-1582).

Gibberellin is a class of diterpenoid plant hormone, designated as GA1 to GAn depending on its kind. The basic structure of Gibberellin consists of 4 rings and contains 20 carbon atoms. Gibberellin plays an essential role in the development and growth of a plant, particularly, in the development of seed, stimulation of germination, growth of a stem, generation and growth of a flower, anthocyanin biosynthesis, fruit-setting, etc (Richard Hooley, *Plant Mol. Biol.*, 1994, 26: 1529-1555). The growth promotion by

gibberellin is not by stimulating the cell division but by stimulating an elongation and enlargement of a cell. In addition, gibberellin stimulates the formation of flower bud or anthesis. And, it is also used for vernalization and long day treatment (Peter Hedden et al., *Annu.Rev.Physiol.Plant Mol. Biol.*, 1997, 48:431-460).

Such functional activities of gibberellin, for example, in the growth of a plant, germination of a dormant seed, promotion of the growth of a dormant bud and parthenocarp in a plant as mentioned above have been widely utilized in farms to increase productivity by accelerating the growth of an herbaceous plant or to increase the size of a fruit.

During the biosynthesis of Giberellin, monooxygenases synthesize  $GA_{12}$  and  $GA_{53}$  in the early stage of the giberrelin synthesis, which are subsequently converted into C19-GA having physiological activities by GA 20-oxidase and  $3\beta$ -hydroxylase resulting in the production of biologically active giberreline. Specifically, GA 20-oxidase oxidizes 20<sup>th</sup> carbons of  $GA_{12}$  and  $GA_{53}$  step by step, providing the backbone structure of C-19 in gibberellin, and  $3\beta$ -hydroxylase catalyzes  $3\beta$ -hydroxylation reaction on the C-19 structure of gibberellin thus obtained by GA 20-oxidase, producing

biologically active gibberellin (Neil Olszewski et al., *Plant Cell*, 2002, 14: S61-S80).

The expression of GA 20-oxidase involved in  
5 biosynthesis of active gibberellin is negatively feedback  
regulated by active gibberellin. Precisely, the level of  
expression of mRNA transcripts for GA 20-oxidase is  
decreased as the active gibberellin synthesis is increased.  
On the contrary, the level of expression of mRNA  
10 transcripts for GA 20-oxidase is increased as the active  
gibberellin synthesis is decreased, resulting in the  
precise regulation of the amount of GA 20-oxidase  
synthesized (Martin et al., *Planta*, 1996, 200(2): 159-66;  
Neil Olszewski et al., *Plant Cell*, 2002, 14: S61-S80;  
15 Victor B. et al., *Plant physiol.*, 2003, 132: 1283-1291).  
For example, *Le20ox-1*, a GA 20-oxidase isolated from  
tomato, converts C-20 gibberellin into C-19 gibberellin  
that is subsequently activated by 3 $\beta$ -hydroxylase. When  
the active gibberellin is over-synthesized, the expression  
20 of *Le20ox-1* mRNA is decreased, by the negative feedback  
regulation, to reduce the amount of *Le20ox-1* enzyme  
synthesized. Thus, C-19 gibberellin synthesis is reduced  
with the decrease of the amount of *Le20ox-1* enzyme  
synthesized, resulting in the regulation of the amount of  
25 active gibberellin (Mariken Rebers et al., *Plant J.*, 1999,

17(3): 241-250). So, low level expression of *Le20os-1* mRNA indicates that active gibberellin is over-synthesized.

It has been reported that *RIN* gene plays a key role  
5 in ethylene synthesis during ripening of tomato fruit.  
Thus, the decrease of the expression of *RIN* gene means the  
decrease of ethylene synthesis during ripening of the  
fruit. That is, the decrease of ethylene synthesis by the  
introduced gene results in the delay of ripening (R.C.  
10 Herner et al., *Plant Physiol.*, 1973, 52: 38-42; Julia  
Vrebalov et al., *SCIENCE*, 2002, 296: 343-346).

At present, the effect of MADS-box genes on the  
development of fruit and seed has not been fully studied.  
15 Although the detailed mechanism of fruit development has  
yet to be found, it has been shown that several MADS-box  
genes are expressed in fruits and seeds. It has been  
recently reported that *AGAMOUS-like 15* (*AGL15*) gene  
directly controls the expression of gene involved in  
20 gibberellin synthesis during seed germination of  
*Arabidopsis thaliana* (Huai Wang et al., *Plant Cell*, 2004,  
16: 1206-1219).

Up to now, a number of researchers have developed  
various transgenic plants by gene manipulation in plants  
25 in order to increase the productivity of crops or to

develop new cultivars. Further, many transgenic plants that have been proved to be safe have been cultivated and allowed on the market for people to consume. Genetic operation in relation to fruits and seeds has the industrial need, since it can increase the productivity of fruit and seed and be applied to the development of a fruit and a horticultural crop by parthenocarpy.

Thus, the present invention has been completed by isolating novel genes that can regulate the development of fruit and seed by inhibiting or promoting the expression of genes which control the synthesis of active gibberellin, and producing a transgenic plant introduced with genes showing enhanced germination efficiency and parthenocarpic fruit formation.

#### **【Disclosure】**

#### **【Technical Problem】**

It is an object of the present invention to provide a novel use of MADS-box gene as a regulator of fruit and seed development.

#### **【Technical Solution】**

In order to achieve the above object, the present invention provides a gene that regulates the fruit and seed development by promoting or inhibiting the expression

of genes that control the synthesis of active gibberellin. The gene is selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a  
5 gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain.

10 The present invention also provides an expression vector containing the above gene.

The present invention further provides transgenic plant cells containing the above gene.

The present invention also provides a transgenic  
15 plant whose fruit and seed development is regulated, produced by regenerating the above transgenic plant cells by tissue culture technique.

The present invention also provides a method of preparation of the transgenic plant.

20 The present invention also provides a regulator for fruit and seed development containing the above gene or a vector harboring the above gene as an effective ingredient.

The present invention also provides a regulator for active gibberellin synthesis containing the above gene or



a vector harboring the above gene as an effective ingredient.

Hereinafter, the present invention is described in  
5 detail.

The present invention provides a gene that can regulates fruit and seed development by promoting or inhibiting the expression of genes that control the synthesis of active gibberellin. The gene is selected from  
10 the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene  
15 encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain.

The gene of the present invention that contains a nucleotide sequence set forth in SEQ. ID. No 1 is  
20 designated '*MdMADS14*' gene (Fig. 1), and the gene having a nucleotide sequence set forth in SEQ. ID. No 2 is designated '*MdMADS16*' gene (Fig. 2). It is preferred that *MdMADS16* gene has 90 ~ 99% sequence homology with *MdMADS14* gene. Also, *MdMADS14* amino acids have at least about 88%  
25 sequence homology with *MdMADS16* amino acids (Fig. 3).

It has been shown that *MdMADS14* gene of the present invention is expressed only in the flowers of an apple tree, but its functions are unknown yet (C.G. van der Linden et al., *Journal of Experimental Botany*, 2002, 53: 1025-1036). Meanwhile, *MdMADS 16* gene of the invention is a novel nucleotide sequence having 90 ~ 99% overall homology with *MdMADS 14* gene (although there is a little difference in homology rate according to a homology comparison program). As shown in Fig. 1 and 2, both *MdMADS16* and 14 genes contain an open reading frame (ORF) coding for 242 amino acids and MADS-domain is located in N-terminal region of each gene. Overall Homology between *MdMADS16* and 14 amino acid sequences is 88.4% while the homology within MADS-domain region is 98.3%, and homology within the region other than MADS-domain is at least 85% (Fig. 3).

Although the gene coding an amino acid sequence having at least 85% homology within the region other than MADS-domain of *MdMADS14* or *MDMADS16* is not represented by a specific sequence list herein, it is easily guessed by a skilled people in this field. *MdMADS16* gene was proved to have at least 85% homology within the region other than MADS-domain and have same functions with *MdMADS14* gene, indicating that a gene coding an amino acid sequence having over 85% homology in the region other than MADS-

domain with the gene of the present invention has equal functions to the gene of the invention.

In the preferred embodiment of the present invention,  
5 *MdMADS14* or *MdMADS16* genes were transferred into a tomato to investigate the function of those genes. From such studies, it has been shown that transformants with over-expressed *MdMADS14* gene resulted in two different phenotypes; one was designated *MdMADS14* sense 1 and the  
10 other *MdMADS14* sense 2. In *MdMADS14* sense 1, ripening of a fruit was delayed but germination of a seed was promoted. Specifically, as shown in Fig. 8 and Fig. 9, ripening of a fruit in *MdMADS14* sense 1 was delayed, compared with that of wild type, while a seed was already germinated in a  
15 fruit, suggesting that germination of a seed was much promoted.

Those characteristics were inherited from T1 generation to T2 generation. As shown in Fig. 10, the seeds (T2 generation) of *MdMADS14* sense 1 were stored at  
20 4 °C within desiccator for one year, and then germination rate was determined and it was found that germination was promoted in T2 generation of *MdMADS14* sense 1, compared with that of wild type. In the case of *MdMADS14* sense 2, parthenocarpic fruit was formed and sepal was developed.  
25 into fruit flesh. Further, anti-sense transformants in

which the expression of *MdMADS14* gene was suppressed showed the common phenotype of not bearing fruit. The above results indicate that *MdMADS14* gene is involved in fruit and seed development.

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Transgenic tomato in which *MdMADS16* gene was over-expressed resulted in also two different phenotypes corresponding to those shown in transformants over-expressing *MdMADS14* gene, each of which was designated  
10 *MdMADS16* sense 1 and *MdMADS16* sense 2. As shown in Fig. 14 ripening of a fruit was delayed in *MdMADS16* sense 1, while a seed was germinated inside a fruit, similar to *MdMADS14* sense 1 shown above. For *MdMADS16* sense 2, as shown in Fig. 15, it showed the same phenotype with *MdMADS*  
15 *14* sense 2 in which sepal was changed into fruit flesh. Further, as shown in Fig. 16, *MdMADS16* anti-sense in which the expression of *MdMADS16* gene was suppressed did not develop a fruit after pollination, like the case of *MdMADS14* anti-sense. The above results indicate that  
20 neither *MdMADS14* anti-sense nor *MdMADS16* anti-sense develop a fruit.

The expressions of *RIN* gene that is required to promote ethylene synthesis during ripening were  
25 investigated in *MdMADS14* senses and *MdMADS16* senses and

were compared with that of wild type. As shown in Fig. 17, the expression level of *RIN* gene in each of *MdMADS14* sense 1 and *MdMADS16* sense 1, both having the phenotype of delayed ripening, was decreased.

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Moreover, the expression level of a gene coding for *LeGA20ox-1* (tomato GA20-oxidase), which can serve as an indicator to indirectly estimate the amount of active gibberellin synthesized, in *MdMADS14* senses and *MdMADS16* senses were compared with that of wild type. As shown in Fig. 18, the expression level of *LeGA20ox-1* gene was much decreased in *MdMADS14* senses and in *MdMADS16* senses than in wild type. Such results indicate that more active gibberellin was synthesized in *MdMADS14* senses and in *MdMADS16* senses than in wild type. On the contrary, the expression level of *LeGA20ox-1* gene was much increased in *MdMADS14* anti-sense and in *MdMADS16* anti-sense than in wild type, as shown in Fig. 19. Those results indicate that active gibberellin was synthesized much less in *MdMADS14* anti-sense and in *MdMADS16* anti-sense than in wild type.

From this study, it has been demonstrated that *MdMADS14* gene and *MdMADS16* gene are highly homologous to each other and are belong to the same gene family.

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Further, through the analysis of gene functions using the transgenic plants, it has also been demonstrated that those genes were involved in fruit and seed development and have activities of promoting or inhibiting active gibberellin synthesis. In conclusion, *MdMADS14* gene and *MdMADS16* gene derived from apple in the present invention are novel having the function of regulating fruit and seed development, which are unknown function in MADS-box genes.

10       The present invention also provides an expression vector containing *MdMADS14* gene or *MdMADS16* gene.

It is preferred that said gene to be inserted into an expression vector is selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain.

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Any conventional expression vector for plant transformation may be used for the insertion of the gene of the present invention. For the present invention, pGA1530 vector for plant transformation was used for the insertion of *MdMADS14* gene (Sung S-K. et al, Plant

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Physiology, 1999, 120:969-978). The pGA1530 vector contains 35S promoter, T7 terminator, and *nptII* (neomycin phosphotransferase), as a marker gene, which provides kanamycin resistance (Stanton B. Gelvin et al, *Plant molecular Biology Manual*, 1988, A3:1-19). For the insertion of *MdMADS16* gene, pCAMBIA 2301 vector for plant transformation was used. The pCAMBIA 2301 vector contains 35S promoter, *nos* (nopaline synthase) terminator, and *nptII* (neomycin phosphotransferase), as a marker gene, that provides kanamycin resistance.

In the preferred embodiment of the present invention, expression vectors were constructed by inserting *MdMADS14* gene into pGA1530 forwardly or reversely. And the constructed vectors were designated 'pMdMADS14' and 'pMdMADS14-R'. Expression vectors were also constructed by inserting *MdMADS16* gene into pCAMBIA 2301 forwardly or reversely, which were designated 'pMdMADS16' and 'pMdMADS16-R'.

The present invention further provides transformed plant cells containing the above gene.

The plant cells that can be used for the introduction of an expression vector of the present invention are not limited to specific forms as long as they can be regenerated into a plant. The cells include, for example, cultured cell buoyant, protoplast, leaf

section and callus. An expression vector of the present invention may be introduced into plant cells by one of conventional methods such as polyethylene glycol method, electroporation, *Agrobacterium* mediating transduction, and particle bombardment, etc. In the preferred embodiment of the present invention, the expression vector was transferred into plant cells by using *Agrobacterium* containing the gene of the present invention, and in particular, *Agrobacterium tumefaciens* LBA4404 (A. Hoekema et al., 1983, *Nature*, 303, 179-181) was used in this invention. *Agrobacterium* containing the gene of the present invention was prepared by transforming the cell with pMdMADS14 or pMdMADS16, expression vectors of the present invention. Each resulting *agrobacterium* was designated '*Agrobacterium tumefaciens* LBA4404/pMdMADS14' and '*Agrobacterium tumefaciens* LBA4404/pMdMADS16', which were deposited with Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on January 30, 2004 (Accession Nos: *Agrobacterium tumefaciens* LBA4404/pMdMADS14; KCTC 10588BP, *Agrobacterium tumefaciens* LBA4404/pMdMADS16; KCTC 10589BP).

The expression vectors of the present invention can be transferred to any kind of plants that can form a fruit or a seed. Particularly, it is preferred that the plant



is selected from a group consisting of food crops such as rice, wheat, barley, corns, soybean, potato, red bean, oat, sorghum; vegetables such as Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, melon, pumpkin, spring onion, onion, carrot; industrial crops such as ginseng, *Acanthopanax senticosus*, tobacco, cotton, sesame, sugar cane, sugar beet, *Perilla japonica*, peanut, rape; fruits such as apple, pear, orange, jujube, peach, kiwifruit, grapes, tangerine, persimmon, plum, apricot, bananas; floricultural crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily, tulip; forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue, perennial ryegrass; fiber crops such as cotton plant; and landscape plants such as flowers and shrubs. It is more preferred that the plant for the introduction of an expression vector of the present invention is tomato plant. In a specific embodiment of the invention, each *Agrobacterium* transformed with pMdMADS14 or pMdMADS16 was added and incubated with cotyledon sections of minitomato to obtain a transformed tomato cotyledon (cells) containing the expression vector of the present invention.

The present invention also provides a transgenic plant, whose fruit and seed development was controlled,

produced by regenerating the above transgenic plant cells by tissue culture technique

Regeneration of the plant cells by tissue culture technique to obtain a transgenic plant of the present invention may be carried out by conventional method of producing transgenic plant. In a specific embodiment of the present invention, tomato cotyledons incubated with agrobacterium having the expression vector containing the gene of the present invention were sub-cultured in a regeneration medium (MS, IAA 1  $\mu$ M, zeatin 10  $\mu$ M, sucrose 3%, cefotaxime 350 mg/l, kanamycin 50 mg/l, agar 0.7%). Shoots produced from the cotyledon sections were then collected and it was further incubated in rooting medium (MS 1/2, IAA 1  $\mu$ M, sucrose 3%, kanamycin 50 mg/l, agar 0.7%), followed by the selection of a transformant. Untransformed shoots underwent necrosis accompanied by color change while transformed shoots were growing normally with roots in. Shoots with roots were transferred to soil and acclimation. Finally, transgenic tomatoes each with the phenotype characterized by that sepal was changed into fruit flesh and parthenocarpic fruit was formed, with the phenotype showing promoted seed germination and delayed ripening, and with the phenotype characterized by that fruit and seed development was inhibited because ovule was not developed, were prepared.

From the above results, it was confirmed that MADS-box genes of the present invention could be effectively used for the regulation of fruit and seed development and parthenocarpic fruit production in plants by promoting or  
5 inhibiting an expression of genes that control the active gibberellin synthesis.

All the plants forming a fruit or a seed can be a target plant of the present invention, which is preferably selected from a group consisting of food crops such as  
10 rice, wheat, barley, corns, soybean, potato, red bean, oat, sorghum; vegetables such as Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, melon, pumpkin, spring onion, onion, carrot; industrial crops such as ginseng, *Acanthopanax senticosus*, tobacco,  
15 cotton, sesame, sugar cane, sugar beet, *Perilla japonica*, peanut, rape; fruits such as apple, pear, orange, jujube, peach, kiwifruit, grapes, tangerine, persimmon, plum, apricot, bananas; floricultural crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily, tulip;  
20 forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue, perennial ryegrass; fiber crops such as cotton plant; and the landscape plants such as flowers and shrubs.

Further, the present invention encompasses the off-  
25 springs and clones of the transgenic plants of the present

invention as well as fruits, seeds, ears, tubers, tuberous roots, column, callus or protoplasts derived therefrom.

The present invention also provides a preparation  
5 method of a plant whose fruit and seed development are regulated, comprising the step of:

A method of preparing a transgenic plant whose fruit and seed development was regulated, comprising the steps of:

- 10 1) Constructing an expression vector comprising the gene according to Claim 1;
- 2) Transferring the vector constructed in Step 1) into Agrobacterium;
- 3) Co-culturing the transformed Agrobacterium of  
15 step 2) with plant tissue; and
- 4) Regenerating the transformed tissue into a mature transgenic plant.

In step 1) of the preparation method of the present invention, a MADS-box gene that regulates fruit and seed  
20 development is preferably selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide  
25 sequence encoding MADS-domain and a gene encoding an amino

acid sequence having at least 85% homology within the region other than MADS-domain. In step 1), any conventional expression vectors for plant transformation may be used for cloning a MADS-box gene. In a specific embodiment, pGA1530 and pCAMBIA 2301 vectors were preferably used for the insertion of *MdMADS14* gene *MdMADS16*, respectively. In step 2), It is preferred that *Agrobacterium* which mediates transferring the gene of the present invention into plant cells is *Agrobacterium tumefaciens* LBA4404 (A. Hoekema et al., 1983, *Nature*, 303, 179-181). It is preferred that *Agrobacterium* having an expression vector containing *MdMADS14* gene or *MdMADS16* gene is '*Agrobacterium tumefaciens* LBA4404/pMdMADS14' (Accession No: KCTC 10588BP) and '*Agrobacterium tumefaciens* LBA4404/pMdMADS16' (Accession No: KCTC 10589BP), respectively.

In step 3), all the plants forming a fruit or a seed can be a target plant of the present invention, which is preferably selected from a group consisting of food crops such as rice, wheat, barley, corns, soybean, potato, red bean, oat, sorghum; vegetables such as Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, melon, pumpkin, spring onion, onion, carrot; industrial crops such as ginseng, *Acanthopanax senticosus*, tobacco, cotton, sesame, sugar cane, sugar

beet, *Perilla japonica*, peanut, rape; fruits such as apple, pear, orange, jujube, peach, kiwifruit, grapes, tangerine, persimmon, plum, apricot, bananas; floricultural crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, 5 lily, tulip; forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue, perennial ryegrass; fiber crops such as cotton plant; and landscape plants such as flowers and shrubs.

10           The present invention also provides a fruit and seed development regulator containing MADS-box gene(s) or an expression vector having the gene(s) as an effective ingredient.

          The present inventors analyzed the function of said 15 genes by examining the phenotypes of *MdMADS14* senses, *MdMADS16* senses and their *anti-senses* in which the genes were suppressed.

          Result from the study showed the following phenotypes: delayed ripening but promoted seed germination 20 (*senses 1*), change of sepal into fruit flesh and formation of parthenocarpic fruits (*senses 2*) and inhibited fruit and seed development (*anti-senses*).

          Particularly, for *senses 1*, germination speed and rate were investigated with seeds (T2 generation) of 25 *senses 1*. As shown in Fig. 9 and Fig. 10, seed

germination was promoted, compared with that of wild type. For senses 2, as shown in Fig. 11, sepal was developed into fruit flesh and parthenocarpic fruit without seed was formed unlike wild type. Plants of anti-senses did not  
5 develop fruits, showing that floral organ development in the early stage was not affect (Fig. 12(a) and (b)) but seed development was inhibited after pollination and so fruits were not formed (Fig. 12(c)).

From the above results, it was confirmed that  
10 *MdMADS14* gene, *MdMADS16* gene and expression vectors containing the genes could be effectively used for the regulation of fruit and seed development in plants

The present invention also provides an active  
15 gibberellin synthesis regulator containing MADS-box gene(s) or an expression vector(s) harboring the gene(s) as an effective ingredient.

*Le20ox-1* is a GA 20-oxidase isolated from a tomato, whose mRNA synthesis is negatively feedback regulated by  
20 active gibberellin. *Le20ox-1* is an enzyme that catalyzes the conversion of C-20 gibberellin into C-19 gibberellin, which subsequently activated by 3 $\beta$ -hydroxylase producing active giberellin. When active gibberellin is synthesized in excess, it negatively regulates the mRNA expression of  
25 *Le20ox-1* by negative feedback, resulting in the decrease

in the production of *Le20ox-1* enzyme. In turn, C-19 gibberellin synthesis is reduced with the decrease in the mRNA expression of *Le20ox-1*, resulting in the regulation of active gibberellin synthesis.

5       The present inventors compared the expressions of *Le20ox-1* gene in *MdMADS14* senses and *MdMADS16* senses in order to investigate the expression levels of the *Le20ox-1* gene in transformed tomatos (Fig. 18 and Fig. 19).

10       The results show that the expression of *Le20ox-1* gene was much decreased in *MdMADS14* senses and in *MdMADS16* senses than in wild type. On the contrary, the expression of *Le20ox-1* gene was increased in *MdMADS14* anti-senses and *MdMADS16* anti-senses. It clearly indicates that the decrease in the expression of *Le20ox-1* gene in *MdMADS14* senses and *MdMADS16* senses, compared with wild type, is caused by negative feedback by over-expressed active gibberellin, while the increase in the expression of *Le20ox-1* gene in *MdMADS14* anti-senses and *MdMADS16* anti-senses results in the decrease in the active gibberellin synthesis. Therefore, it was confirmed that *MdMADS14* and *MdMADS16* genes of the present invention and a vector containing those genes could be effectively used for the control of active gibberellin synthesis.

25       【Description of Drawings】



Fig. 1 is a schematic diagram showing the nucleotide sequence of *MdMADS14* gene containing a nucleotide sequence coding for MADS-domain and K-domain as well as the amino acid sequence encoded thereby.

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Fig. 2 is a schematic diagram showing the nucleotide sequence of *MdMADS16* gene containing a nucleotide sequence coding for MADS-domain and K-domain as well as the amino acid sequence encoded thereby.

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Fig. 3 is a schematic diagram showing the homology between amino acid sequences of *MdMADS14* and *MdMADS16*.

Fig. 4 is a phylogenetic tree showing the relation of *MdMADS14* and *MdMADS16* gene to other MADS-box genes in the same family. AP1: APETALA1 sub-family, SQUA: SQUAMOSA sub-family, AGL2: AGAMOUS-LIKE2 sub-family, AGL6: AGAMOUS-LIKE6 sub-family, AG: AGAMOUS sub-family.

Fig. 5 is a set of graphs showing the results from real-time PCR in which the mRNA level of *MdMADS14* and *MdMADS16* genes in each developmental stage of leaf, reproductive organ and fruit are quantified and compared.

A: Result of real-time PCR with cDNA from leaves of an apple,

25

B: Result of real-time PCR with cDNA from flower bud of an immature apple,

C: Result of real-time PCR with cDNA from flower of a mature apple,

5 D: Result of real-time PCR with cDNA from fruit of an immature apple,

E: Result of real-time PCR with cDNA from fruit of a mature apple,

10 F: A graph showing the melting temperature of each PCR product, obtained through melting curve analysis during real-time PCR,

G: A graph showing the expression profile of *MdMADS14* gene in each organ and in each developmental stage based on the results of graphs A - E,

15 H: A graph showing the expression profile of *MdMADS16* gene in each organ and in developmental stage based on the results of graphs A - E.

a - leaves;

20 b - flower buds with developing floral organ primordia;

c - flower buds with developing anthers and fused carpels;

d - fruits (7 to 10 days after pollination);

e - ripe fruit.

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Fig. 6 is a set of photographs showing the expression profiles of *MdMADS16* mRNA in floral organ and fruit developmental stages of an apple tree by in situ hybridization.

- 5           A: Young flower bud with inflorescence meristem,  
           B: Young flower bud at the very early stage of differentiation into floral organ having floral primordium,  
           C: Flower bud in the process of differntiating into floral organ,
- 10           D: Immature fruit at 7-10 days after pollination,  
           c - carpel primordium;  
           ft - floral tube;  
           im - inflorescence meristem;  
           l - leaf appendage;
- 15           o - ovule;  
           st - stamen primordium

Fig. 7 is a photograph showing the result of PCR amplifying *npt II* gene using genomic DNA of a transformant, in order to confirm the insertion of a recombinant *pMdMADS14* plasmid in a transgenic tomatos.

- 20           Lane a: Wild type,  
           Lane b: *MdMADS14* anti-sense,  
           Lane c: *MdMADS14* sense 1,
- 25           Lane d: *MdMADS14* sense 2

Fig. 8 is a photograph showing the fruit of T1 generation of *MdMADS14* sense 1 in which ripening is delayed, compared with wild type.

5        Fig. 9 is a set of photographs showing the comparison of germination rate between sense 1 seeds (T2 generation), shown in Fig. 8, and wild type seeds, which had been cultured for 5 days under the same conditions.

10       Fig. 10 is a set of photographs showing the comparison of germination rate between sense 1 seeds (T2 generation), shown in Fig. 8, and wild type seeds. Before the comparison, seeds of both groups were collected and stored in cool dry condition for one year, and then they  
15       were cultured for 5 days under the same conditions.

Fig. 11 is a set of photographs showing the result of comparison of fruits between *MdMADS14* sense 2 and wild type.

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Fig. 12 is a set of photographs showing the results of *MdMADS14* anti-sense phenotype analysis. Fig. 12(a) shows the overall appearances of *MdMADS14* anti-sense and wild type, Fig. 12(b) is a histological observation of  
25       flower and carpel before pollination, and Fig. 12(c) is a

histological observation of early fruit and ovule after pollination.

Fig. 13 is a photograph showing the result of PCR  
5 amplifying *nptII* gene using genomic DNA of a transformant,  
in order to confirm the insertion of a recombinant  
*pMdMADS16* plasmid in a transgenic tomatos.

Lane a: Wild type,  
Lane b: *MdMADS16* anti-sense,  
10 Lane c: *MdMADS16* sense 1,  
Lane d: *MdMADS16* sense 2

Fig. 14 is a set of photographs showing that  
*MdMADS14* sense 1 and *MdMADS16* sense 1 have the common  
15 phenotype of early seed germination inside of a fruit,  
compared with wild type.

Fig. 15 is a set of photographs showing that  
*MdMADS16* sense 2 and *MdMADS14* sense 2 have the common  
20 phenotype that sepal is changed into fruit flesh and  
pathenocarpic fruit is formed as shown in each cross  
section of fruit.

Fig. 16 is a set of photographs showing overall  
25 appearances of *MdMADS16* anti-sense and wild type (upper),

and a magnified view thereof (lower) indicating no fruit formation.

Fig. 17 is a set of photographs showing the expression levels of *RIN* gene in the fruits of *MdMADS14* senses and *MdMADS16* senses, which were quantified by RT-PCR and PCR-Southern blot.

Lane a: Wild type,  
Lane b: Fruit of *MdMADS14* sense 1,  
10 Lane c: Fruit of *MdMADS16* sense 1,  
Lane d: Fruit of *MdMADS14* sense 2,  
Lane e: Fruit of *MdMADS16* sense 2

Fig. 18 is a set of photographs showing the expression levels of *Le20ox-1* gene in the fruits of *MdMADS14* senses and *MdMADS16* senses, which were quantified by RT-PCR and PCR-Southern blot.

Lane a: Wild type,  
20 Lane b: Fruit of *MdMADS14* sense 1,  
Lane c: fruit of *MdMADS16* sense 1,  
Lane d: Fruit of *MdMADS14* sense 2,  
Lane e: fruit of *MdMADS16* sense 2

Fig. 19 is a set of photographs showing the expression levels of *Le20ox-1* gene in leaves of *MdMADS14* senses, anti-senses, *MdMADS16* senses and anti-senses, which were quantified by RT-PCR and PCR-Southern blot.

- 5           Lane a: Wild type,  
          Lane b: Leaves of *MdMADS14* sense 1,  
          Lane c: Leaves of *MdMADS16* sense 1,  
          Lane d: Leaves of *MdMADS14* sense 2,  
          Lane e: Leaves of *MdMADS16* sense 2,  
10          Lane f: Leaves of *MdMADS14* anti-sense,  
          Lane g: Leaves of *MdMADS16* anti-sense

【Best Mode】

15          Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

          However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

20          <Example 1> Cloning of an MADS-box gene from apple and nucleotide sequence analysis thereof

          cDNA library (SUNG. S.K. et al., *Mol. cells*, 8: 565-577, 1998) constructed from pistil of apple flower (*Malus domestica* Borkh cv. Fuji) was used for the preparation of

25

cDNA pool by *in vivo* excision (Stratagene, USA, Catalog #200450). The nucleotide sequence of MADS-box genes was searched from GenBank (NIH, USA), a database of nucleotide sequences of genes, and then a set of forward and reverse primer was designed from the most conservative region of the nucleotide sequence, which are represented by SEQ. ID. No 3 and SEQ. ID. No 4, respectively. The degenerate PCR was then carried out on pistil cDNA pool, constructed from apple flowers, as a template using the forward and reverse primer set.

The PCR reaction mixture contains 100 ng of cDNA template, 10  $\mu$ M of each forward and reverse primer, 20 mM of Tris-HCL (pH 8.4), 50 mM of KCl, 1.5 mM of  $MgCl_2$ , 0.2 mM of each of dNTP, and 2.5 unit of *taq* polymerase (*Thermus aquaticus* DNA polymerase) (TaKaRa, Japan). The PCR reaction was carried out on a thermal cycler (Perkin Elmer 9600, Perkin Elmer) under the following cycling condition: initial denaturation at 94°C for 3 min; 1 cycle of denaturation, annealing and extension at 94°C/30 seconds, 60°C/1 minute, and 72°C/1 minute, respectively; followed by a touch down PCR where the annealing temperature is decreased by 1°C every cycle to 50°C (touchdown annealing temperature) At the last annealing temperature (50°C), 10 cycles were performed, and final extention at 72°C for 5 minutes to complete the



PCR processes. The resulting PCR product was then diluted 10 fold and used as a template for subsequent nested degenerated PCR using forward primer set forth in SEQ. ID. No 5 and reverse primer set forth in SEQ. ID. No.4 The PCR reaction was carried out under the same condition as above The PCR product was cloned into pGEM-T Easy vector (Promega, USA) and then nucleotide sequence of the product was investigated by using T7 primer and SP6 primer.

10       The resulting nucleotide sequence data was then analyzed by homology analyzing program (blast, NCBI, USA) and was found to be a partial sequence of the MADS-box gene. Subsequently, a reverse primer set forth in SEQ. ID. No 7 based on the nucleotide sequence information of the above, and a forward primer set forth in SEQ. ID. No 6 based on the nucleotide sequence containing restriction enzyme site of pBluescriptSK(-) vector (Stratagene, USA) were then designed and used for a subsequent PCR reaction. The PCR in the same reaction buffer as above was performed under the following cycling condition; initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation, annealing and extension at 94°C/30 seconds, 60°C/1 minute, and 72°C/1 minute, respectively; and final extension at 72°C for 5 minutes to complete the reaction. 25 A 450 bp PCR product thus obtained was then cloned into a

pGEM-T Easy vector and the nucleotide sequence of the cloned fragment was investigated by using T7 primer and SP6 primer. The nucleotide sequences data thus obtained were analyzed by homology analyzing program (blast, NCBI, USA) and found to correspond to 5' end of MADS-box gene having 687bp. Subsequently, based on the resulting sequence information, a forward primer represented by SEQ. ID. No 8 and reverse primer represented by SEQ. ID. No 9 were designed for a subsequent PCR. The PCR in the same reaction buffer as above was performed under the following cycling condition; initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation, annealing and extension at 94°C/30 seconds, 60°C/1 minute, and 72°C/1 minute, respectively; and final extension at 72°C for 5 minutes to complete the reaction. An amplified product about 910 bp in size thus obtained was then cloned into a pGEM-T Easy vector. The resulting plasmid was then used for a transformation of XL1-Blue MRF', an *Escherichia coli* strain, and then isolated therefrom.

Nucleotide sequence of the isolated plasmid was investigated. As a result, full-length sequences of two different genes, as shown in Fig. 1 and Fig. 2, were identified which were highly homogenous but had different amino acid sequence.

The full-length sequences thus obtained were each represented by SEQ. ID. No 1 (Fig. 1) and by SEQ. ID. No 2 (Fig. 2). Further, the nucleotide sequence set forth in SEQ. ID. No 2 were then analyzed by clustal method using MegAlign (DNASTar Inc., USA), a program for sequence analysis and was confirmed to be a novel gene having different nucleotide sequence from that represented by SEQ. ID. No 1. As shown in Fig. 1, the gene set forth in SEQ. ID. No.1 contains an open reading frame (ORF) encoding 242 amino acids, and also a nucleotide sequence encoding MADS-domain at its N-terminal region. Accordingly, it was designated '*MdMADS14*'. As shown in Fig. 2, the gene set forth in SEQ. ID. No 2, which was designated '*MdMADS16*', also contains an ORF encoding 242 amino acids of the same size as the one in *MdMADS14* gene, and also a nucleotide sequence encoding MADS-domain at its N-terminal region. The homology between *MdMADS14* and *MdMADS16* genes in 242 amino acid sequences was 88.4%, the homology between the two genes in MADS-domain region was 98.3%, and the homology between the two genes in the region other than MADS-domain was over 85% (Fig. 3). High homology of MADS-domain regions seems to be common among the members of MADS-box gene family, thus a gene having homology with *MdMADS14* and *MdMADS16* gene has to be identified based on the homology data from the region other than MADS-domain.

Thus, it appears that *MdMADA14* gene and *MdMADS16* gene are members of the same family. Further it was found that, as shown by the phylogenetic tree of MADS-box genes (Fig. 4), these two genes are more closely related to each other than to any other gene of MADS-box gene group.

<Example 2> Analysis of Expression of *MdMADS14* and *MdMADS16* genes in various organs and in each different developmental stage using real-time PCR

Real-time PCR was performed to investigate the expressions of *MdMADS14* gene and *MdMADS16* gene in a variety of organs and in various developmental stages of fruits. Test samples were taken from various parts of an apple tree, i.e., leaves, flower buds, flowers, young fruit at 2 weeks after blooming and at 24 weeks after blooming. Total RNA was extracted from each of the above samples and used for real-time PCR was performed with it.

The expression levels of the genes in each organ and in developmental stage were quantified by real-time PCR using SYBR Green Master Mix Kit (Applied Biosystems, USA) as recommended by the supplier. Specifically, the first strand cDNA pool was constructed from 2  $\mu$ g of total RNA extracted from each of the samples above. Primer concentrations were first optimized such that non-specific PCR products or a primer dimer was not formed during the PCR. The optimum concentration determined for each primer

was as follows: for *MdMADS14* gene amplification, forward primer of SEQ. ID. No 10 and reverse primer of SEQ. ID. No 11 were 50 nM and 300 nM, respectively; for *MdMADS16* amplification, forward primer of SEQ. ID. No 12 and reverse primer of SEQ. ID. No 13 were both 900nM; and for actin gene amplification as an internal control, forward primer of SEQ. ID. No 14 and reverse primer of SEQ. ID. No 15 were both 900 nM.

Each primer set was designed such that the size of amplified products is not more than 120 bps for accurate measurement within a short reaction time possible. Real-time PCR was performed on the first strand cDNA pool from each organ and developmental stage using a primer set specific for each gene to be detected at optimum concentrations as described above. The PCR was carried out on a thermal cycler for real-time PCR (7300 Real-time PCR system, Applied Biosystems, USA) under the following cycling condition: initial incubation at 50°C for 2 minutes to activate polymerase therein; followed by denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 15 seconds and hybridization or annealing at 60°C for 1 minute. The signal intensity from fluorescent dye in each amplified product was detected in a hybridization or annealing step of every cycle of RT-PCR to quantify the PCR product amplified. The expressions

levels of *MdMADS14* gene and *MdMADS16* gene in each organ and developmental stage were then determined and normalized against the amount of actin synthesized.

As shown in Fig. 5, the expression of *MdMADS14* gene was observed from flower buds to the young fruit development stage, while it is rarely expressed in leaves and mature fruits of an apple tree. The expression pattern of *MdMADS16* gene was similar to the one shown by *MdMADS14* gene, only the expression levels of *MdMADS16* gene were lower than that of *MdMADS14* gene. In order to examine specificity of each reaction product, melting curve analysis was performed. As a result, *MdMADS14* and *MdMADS16* were confirmed to be different from each other, showing different melting temperature.

#### <Example 3> mRNA Expression of *MdMADS16* gene by *in situ* hybridization

The expression of mRNA of *MdMADS16* gene in young flower bud and flowers after pollination from an apple tree was analyzed by *in situ* hybridization. The sample cut at appropriate size was fixed in a fixing solution, and embedded in paraffin block. The paraffin block was then sectioned at 10  $\mu$ m thick followed by hybridization with an appropriate nucleotide probe. The probe used

herein was directed to a region other than MADS-domain and K-domain that are commonly found in MADS-box genes, and was prepared by labeling the antisense strand of *MdMADS16* gene with DIG. The probe represented by SEQ. ID. No 16 was preferably used in this invention. However, the difference between the two genes was not distinguished by the probe, because homology in those regions between *MdMADS16* gene and *MdMADS14* gene was more than 88% (88.1%). In conclusion, as shown in Fig. 6, *MdMADS16* gene was strongly expressed in inflorescence meristem and in ovule. The result indicates that *MdMADS16* gene is involved in fruit and seed development and flower organ formation.

<Example 4> Generation of a transgenic plant containing *MdMADS14* gene

DNA isolated from apple containing *MdMADS14* gene as in Example 1 was digested with *Pst*I and *Hind*III, then the resulting fragment was cloned into a pBluescriptSK(-) vector (Stratagene, USA) digested with the same enzymes.

A recombinant vector for over-expression was constructed by digesting it with *Xba*I and *Cla*I and then cloning forwardly into pGA1530 vector, a vector for plant transformation, pre-digested with the same enzymes. A recombinant vector for the inhibition of gene expression was also constructed by digesting recombinant pBluescriptSK(-) vector containing *MdMADS14* gene with

XbaI and HindIII, and cloning it reversely into pGA1530 vector, a vector for plant transformation, pre-digested with the same enzymes. The pGA1530 vector for plant transformation contains 35S promoter, T7 terminator and  
5 nptII (neomycin phosphotransferase) as a selection marker which provides kanamycin resistance (*Plant molecular Biology Manual*, 1988, A3:1-19). The resulting recombinant plasmid containing MdMADS14 gene in forward and reverse direction was designated 'pMdMADS14', and 'pMdMADS14-R',  
10 respectively. The recombinant plasmids were then used to transform *Agrobacterium tumefaciens* LBA4404 (A. Hoekema et al., 1983, *Nature*, 303, 179-181), and each transformed *Agrobacterium tumefaciens* containing 'pMdMADS14' or 'pMdMADS14-R' was selected in a growth medium containing  
15 Kanamycin, which were deposited with Korean Collection for Type Cultures (KCTC) of Korea Institute of Bioscience and Biotechnology (KRIBB) on January 30, 2004 (Accession No: KCTC 10588BP).

Minitomato (*Lycopersicon esculentum* cv. 'Micro-Tom')  
20 was used for the generation of a transgenic plant into which pMdMADS14 was transferred. In order to select a target tissue, mature seeds of minitomato were first sterilized by immersion in 70% ethanol for 1 minute, washing with sterilized water three times, immersion in 2%  
25 NaOCl (Sodium hypochloride) for 15 minutes, followed by



washing with sterilized water more than 7 times. The seeds were then transferred to a plate containing seed germination medium (1/2 MS, 3% sucrose, 0.8% agar). 14 days later, seeds began to be germinated. As cotyledon was opened and true leaf began to be sprout, the cotyledon was selected as a target tissue for transformation.

The recombinant *Agrobacterium tumefaciens* LBA4404, prepared as in the above Example, was cultured in YEP medium (1% yeast extract, 1% peptone, 0.5% NaCl) containing 50 mg/l of kanamycin until OD<sub>600</sub> was 0.8. The cultured *Agrobacterium* was co-cultured in 1/2 MS medium supplemented with 200 µM of acetosyringone with shaking at 150 rpm, at 22°C for 2 hours. Then, the target tissue, cotyledon section of a minitomato prepared as above, was added thereto and co-cultured with shaking at 150 rpm, at 22°C for 10 minutes. The resulting cotyledon sections were then placed onto a plate containing co-culture medium (MS, IAA 1 µM, zeatin 10 µM, sucrose 3%, acetosyringone 200 µM, agar 0.7%) and cultured for 2 days. Then, the cotyledon sections were transferred onto a regeneration medium (MS, IAA 1 µM, zeatin 10 µM, sucrose 3%, cefotaxime 350 mg/l, kanamycin 50 mg/l, agar 0.7%), followed by two sub-cultures in succession at three weeks interval. Then, shoots generated from the cotyledon sections were

transferred onto selective root-inducing medium (MS 1/2, IAA 1  $\mu$ M, sucrose 3%, kanamycin 50 mg/l, agar 0.7%) containing kanamycin to select transformants. The transformed shoots can be differentiated from untransformant since the untransformant undergoes necrosis accompanied by color change while transformed shoots were growing normally with roots. Shoots with roots were then transferred onto soil after acclimatization.

As a result, transformants over-expressing *MdMADS14* gene (senses) showed two phenotypes, each of which was designated "transformant 1" (*MdMADS14* sense 1) and transformant 2 (*MdMADS14* sense 2). Another transformant in which the expression of *MdMADS14* gene was suppressed (anti-sense) showed the phenotype of no-fruit formation, which was designated "inhibited transformant 1" (*MdMADS14* anti-sense). As shown in Fig. 7, the successful integration of *MdMADS14* gene into the genome of each of the above transformant was verified by PCR amplifying *nptII* gene in each transformant. A set of forward and reverse Primers used for the PCR to confirm the integration of *nptII* gene is represented by SEQ. ID. No 17 and by SEQ. ID. No 18, respectively.

<Example 5> Analysis of the functions of *MdMADS14* gene in a transgenic plant

Phenotypes of *MdMADS14* senses and anti-sense, prepared in the above example 4, were investigated to analyze the functions of *MdMADS14* gene in plants.

As a result, *MdMADS14* senses showed two different phenotypes; sense 1 showed delayed ripening but promoted seed germination and sense 2 showed the characteristics of that sepal was changed into fruit flesh and parthenocarpic fruit was formed.

As shown in Fig. 8 and Fig. 9, sense 1 showed delayed fruit ripening, comparing to wild type, but promoted seed germination. Such characteristics were observed not only in T1 generation but also in T2 generation, indicating that the characteristics are inherited.

In order to confirm the above result, germination speed and germination rate of seed (T2 generation) of sense 1 were measured (Table 1, measurement of the primary germination rate). Specifically, 25 seeds each from wild type tomato and *MdMADS14* sense 1 as shown in Fig. 8 were harvested, and then dried for 10 days. Subsequently, the seeds were placed on a filter paper moisten with distilled water in a petridish and cultured at 25°C for 5 days. Germination speed for each group was measured and statistically analyzed. As shown in Fig. 9, seed germination in sense 1 was much promoted than in wild type.

As shown in Table 1, while the average germination speed of seeds in sense 1 was 1.77 day, the average germination speed of seeds in wild type was 3.8 days. Moreover, germination rate of seed in sense 1 was twice as high as the wild type. Wild type seeds and seeds (T2 generation) of sense 1 were stored in cool/dry condition for a year, then germination speed and germination rate were investigated again by the same method as used above (Table 2, measurement of the second germination rate). As a result, as shown in Fig. 10, seed germination speed and germination rate in sense 1 was still promoted, comparing to wild type. As shown in Table 2, the average germination speed of sense 1 seeds was 3 days, while the average germination speed of wild type tomato seeds was 6 days, indicating that germination speed was shortened in sense 1 twice as much as in wild type, and germination rate in sense 1 was still two-fold higher than in wild type. After one-year storage, germination speed and germination rate were slow and dull in general, but the speed and rate in sense 1 were still about two-fold higher than those in wild type.

【Table 1】

	Seeds from Transformant 1 (T2)	Seeds from non- transformant
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Average Germination Term	1.77 Days	3.8 Days
Germination Rate	100%	50%

【Table 2】

	Seeds from Transformant 1 (T2) ; 1 year storage in a cool/dry place.	Seeds from Non-transformant ; 1 year storage in a cool/dry area
Average Germination Term	3Days	6Days
Germination Rate	92%	40%

*MdMADS14* sense 2 showed such phenotype as sepal was changed into fruit flesh and parthenocarpic fruit was formed. As shown in Fig. 11, sepal in sense 2 was changed into fruit flesh with being corpulent and parthenocarpic fruit was formed without seed formation, unlike wild type.

In the meantime, *MdMADS14* anti-sense plant showed common phenotype that was characterized by no-fruit formation. As shown in Fig. 12, the development of floral organ in the early stage was not affected (Fig. 12(a) and Fig. 12(b)) but seed development after pollination was inhibited, and so fruits were not grown enough (Fig. 12(c)). Floral organ and ovary of each developmental stage in anti-sense were fixed in a fixing solution (50mM PIPES; pH 6.8, 4% paraformaldehyde), leading to plastic

embedding. The samples were cut by 7  $\mu$ m and stained with toluidine blue O, followed by histological observation.

As a result, as shown in Fig. 12(b) and 12(c), the development of ovule in early developmental stage of floral organ was not much affected, but the development of ovule and ovary after pollination was clearly inhibited.

<Example 6> Generation of a transgenic plant having MdMADS16 gene and analysis of the functions of MdMADS16 gene in the plant

DNA isolated from apple containing *MdMADS16* gene as in Example 1 was digested with *Pst*I and *Hind*III and the resulting fragment was cloned into a pBlue-scriptSK(-) vector (Stratagene, USA) digested with the same enzymes. The vector was digested with *Bam*HI and *Hind*III again, followed by fill-in with the fragment containing the above gene. Then, *MdMADS16* gene was sub-cloned into pRTL2 vector (Restrepo, M. et al., *Plant Cell*, 1990, 2: 987-998) pre-digested with *Sma*I. The plasmid prepared by the above procedure included both forward cloning and reverse cloning of *MdMADS16* gene, so both forwardly cloned *MdMADS16* gene and reversely cloned *MdMADS16* gene were separated through nucleotide sequencing. Each of them was digested with *Hind*III, followed by cloning into pCAMBIA 2301 vector (CAMBIA, Australia) for plant transformation,

which was pre-digested with the same enzyme. pCAMBIA 2301 vector for plant transformation contains 35S promoter, *nos* (*nopaline synthase*) and *npt II* (*neomycin phosphotransferase*) as a marker gene, which provides  
5 kanamycin resistance. The recombinant plasmid in which *MdMADS 16* was forwardly inserted was designated 'pMdMADS16' and the recombinant plasmid in which *MdMADS16* gene was reversely inserted was designated 'pMdMADS16-R'. Those recombinant plasmids were inserted in *Agrobacterium*  
10 *tumefaciens* LBA4404, and then strains containing those recombinant plasmids, pMdMADS16 and pMdMADS16-R, were selected from kanamycin selection medium. The selected *Agrobacterium tumefaciens* containing the recombinant plasmid pMdMADS16 was deposited at Korean Collection for  
15 Type Cultures (KCTC) of Korea Institute of Bioscience and Biotechnology (KRIBB) on January 30, 2004 (Accession No: KCTC 10589BP), which was further used for *Agrobacterium tumefaciens*-mediated transformation of minitomato plant as described in Example 3. *MdMADS16* senses, transgenic  
20 minitomato plant generated by the above procedure, showed two phenotypes and was designated *MdMADS16 sense 1* and *MdMADS16 sense 2* depending on their phenotypes. The phenotypes were the same as those of *MdMADS14* senses. In other words, *MdMADS16 sense 1*, as shown in Fig. 14, showed  
25 delayed ripening but promoted seed germination like

*MdMADS14* sense 1. *MdMADS16* sense 2, as shown in Fig. 15, showed the phenotype of transformation of sepal into fruit flesh as shown in Fig. 16 like *MdMADS14* sense 2. *MdMADS16* anti-sense showed phenotype of no-fruit formation after pollination, like *MdMADS14* anti-sense, which indicate that both *MdMADS14* gene and *MdMADS16* genes are involved in fruit formation.

From the Examples above, it can be known that *MdMADS14* gene and *MdMADS16* gene belong to the same family, showing high homology, and are involved in seed and fruit development from the functional analysis of transgenic plants. Such functions of *MdMADS14* and *MdMADS16* gene of the present invention are novel, which has not been found previously in currently known MADS-box genes.

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<Example 7> Analysis of the expressions of *RIN* gene in *MdMADS14* and *MdMADS16* senses by RT-PCR

Both *MdMADS14* sense 1 and *MdMADS16* sense 1 generated in Example 4 and Example 6, respectively, show the same phenotype as was shown for the tomato plant in which the ethylene synthesis is inhibited during ripening (Julia Vrebalov et al., *SCIENCE*, 2002, 296: 343-346). Thus, *RIN* gene expression that plays an important role in ethylene synthesis during ripening of tomato was investigated in



*MdMADS14* sense 1 and *MdMADS16* sense 1 by reverse transcription-polymerase chain reaction (RT-PCR).

Specifically, the first strand cDNA pool was prepared from each 2  $\mu$ g of total RNA extracted from fruits of senses and wild type using reverse transcriptase primed with oligo(dT) primer. RT-PCR was then performed by using the cDNA pool as a template according to the method known in the pertinent art. Particularly, the PCR conditions were set to assure the validity of the analysis such that the amplified products are in exponential phase. Actin primer, used in the above example 2, was used for internal control.

In order to confirm whether or not an introduced gene was expressed in a transformant, forward primer represented by SEQ. ID. No 19 and reverse primer represented by SEQ. ID. No 20 were prepared based on common nucleotide sequence region (referred as *MdMADS*) between *MdMADS14* gene and *MdMADS16* gene, and then used for the amplification of *MdMADS*. Forward primer represented by SEQ. ID. No 21 and reverse primer represented by SEQ. ID. No 22 were used for the amplification of *RIN* gene. PCR was performed under the following cycling condition: initial denaturation at 95°C for 10 minutes; 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute; and final extension at 72°C for 10 minutes. The obtained PCR

products were then electrophoresed on an agarose gel, and the gel was transferred onto a Hybond N+ membrane according to a method known in the pertinent art. The membrane was then allowed to hybridize with the probe for *RIN* gene, *MdMADS* gene and actin gene, each of which was labeled with biotin, and the signals from each probe hybridized to its corresponding target sequence were detected using Southern-Star™ System (Applied biosystems, USA) recommended by the supplier.

The results are shown in Fig. 17. First, the successful expression of the gene introduced into each transformant was confirmed by the signals detected only in transformants but not in a non-transformant. Further, the expression of *RIN* gene was reduced in *MdMADS14 sense 1* and *MdMADS16 sense 1*, which is consistent with delayed ripening. On the contrary, the expressions of *RIN* gene in *MdMADS14 sense 2* and *MdMADS16 sense 2* that forms parthenocarpic fruit were not much different from that of wild type.

It has been known that reduced expression of *RIN* gene represents the reduced ethylene synthesis during ripening since *RIN* gene plays an essential role in ethylene synthesis during ripening (R.C. Herner et al., *Plant Physiol.*, 1973, 52: 38-42; Julia Vrebalov et al., *SCIENCE*, 2002, 296: 343-346). Therefore, the results from

this experiment indicate that the delayed ripening in both *MdMADS14* sense 1 and *MdMADS16* sense 1 was resulted from the decrease of ethylene synthesis by the introduction of *MdMADS14* and *MdMADS16* gene. Further, it indicates that *MdMADS14* gene and *MdMADS16* gene of the present invention can be effectively used for the regulation of fruit development and fruit ripening delay.

<Example 8> Analysis of the expression of *Le20ox-1* gene in a transformant harboring *MdMADS14* gene or *MdMADS16* gene by RT-PCR

In order to examine the level of active gibberellin synthesized in *MdMADS14* senses and *MdMADS16* senses obtained in Example 4 and Example 6, the expression level of *Le20ox-1* gene was investigated. As mentioned hereinbefore, the mRNA synthesis of *Le20ox-1* gene, which is involved in the conversion of C-20 into C-19 gibberellin followed by activation by 3 $\beta$ -hydroxylase leading to active gibberellin, is regulated by negative feedback from high level of active gibberellin present in a cell. Thus, the mRNA level of *Le20ox-1* gene is negatively correlated with the amount of active gibberellin present.

The expression levels of *Le20ox-1* gene in fruits and leaves from transgenic plants and wild type were compared

and determined by RT-PCR followed by Southern blot as described in Example 7 except that, for the amplification of *Le20ox-1* gene, forward primer represented by SEQ. ID. No 23 and reverse primer represented by SEQ. ID. No 24 were used.

The results are shown in Fig. 18. The expression of *Le20ox-1* gene in fruits of *MdMADS14* senses and *MdMADS16* senses were significantly decreased, compared with that of wild type, which suggests that synthesis of active gibberellin in *MdMADS14* senses and *MdMADS16* senses was much increased, comparing to that in wild type.

The expression levels of *Le20ox-1* gene in leaves of *MdMADS14* senses, *MdMADS16* senses, *MdMADS14* anti-sense and *MdMADS16* anti-sense were also compared. The results are shown in Fig. 19. The expression of *Le20ox-1* gene were significantly decreased in leaves of *MdMADS14* senses and *MdMADS16* senses, compared with wild type, but the expression of *Le20ox-1* gene were increased in leaves from *MdMADS14* anti-sense and *MdMADS16* anti-sense. The results indicate that synthesis of active gibberellin in *MdMADS14* senses and *MdMADS16* senses was much increased, but synthesis of active gibberellin in *MdMADS14* anti-sense and *MdMADS16* anti-sense was much decreased, comparing to that in wild type.

Therefore, *MdMADS14* gene and *MdMADS16* gene of the present invention can be effectively used for the regulation of active gibberellin synthesis.

5     **【Industrial Applicability】**

The genes of the present invention regulate fruit and seed development by inhibiting or promoting active gibberellin synthesis. A transgenic plant containing the gene of the present invention exhibits phenotype that is characterized by promoted seed development, transformation of calyx into fruit flesh and formation of parthenocarpic fruit. Thus, the genes of the present invention can be effectively used for the regulation of active gibberellin synthesis, production of parthenocarpic fruit, and fruit and seed development, all of which are important in increasing the productivity of crops.

**【Sequence List Text】**

The SEQ. ID. No 1 is a nucleotide sequence of *MdMADS14*,

The SEQ. ID. No 2 is a nucleotide sequence of *MdMADS16*,

The SEQ. ID. No 3, No 4 and No 5 are nucleotide sequences of degenerate primers used for degenerate PCR described in Example 1,

The SEQ. ID. No 6 and No 7 are nucleotide sequences of primers used for PCR to amplify the nucleotide sequence of SEQ. ID. No 1 in Example 1,

5 The SEQ. ID. No 8 and No 9 are nucleotide sequences of primers used for PCR to amplify the nucleotide sequence of SEQ. ID. No 1 in Example 2,

The SEQ. ID. No 10 and No 11 are nucleotide sequences of primers used for the amplification of *MdMADS14* gene by real-time PCR and RT-PCR described in  
10 Example 2,

The SEQ. ID. No 12 and No 13 are nucleotide sequences of primers used for the amplification of *MdMADS16* gene by real-time PCR and RT-PCR described in Example 2,

15 The SEQ. ID. No 14 and No 15 are nucleotide sequences of primers used for the amplification of *actin* gene by real-time PCR and RT-PCR described in Example 2,

The SEQ. ID. No 16 is a nucleotide sequence of probe used in *in situ hybridization* described in Example 3,

20 The SEQ. ID. No 17 and No 18 are nucleotide sequences of primers used for PCR to confirm the expression of *nptII* gene described in Example 4,

The SEQ. ID. No 19 and No 20 are nucleotide sequences of primers used for real-time PCR and RT-PCR for  
25 the amplification of *MdMADS* gene described in Example 7,

The SEQ. ID. No 21 and No 22 are nucleotide sequences of primers used for real-time PCR and RT-PCR for the amplification of *RIN* gene described in Example 7,

5 The SEQ. ID. No 23 and No 24 are nucleotide sequences of primers used for real-time PCR and RT-PCR for the amplification of *Le20ox-1* gene described in Example 8.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis  
10 for modifying or designing other embodiments for carrying out the same purposes as the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and  
15 scope of the invention as set forth in the appended claims.